Molecular Biology of the Cell
Vol. 16, 3117–3127, July 2005

Protein Kinase A Gating of a Pseudopodial-located RhoA/ROCK/p38/NHE1 Signal Module Regulates Invasion in Breast Cancer Cell Lines[D]

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Submitted October 29, 2004; Revised April 1, 2005; Accepted April 12, 2005
Monitoring Editor: John Cleveland

Metastasis results from a sequence of selective events often involving interactions with elements of the tumor-specific physiological microenvironment. The low-serum component of this microenvironment confers increased motility and invasion in breast cancer cells by activating the Na^+/H^+ exchanger isoform 1 (NHE1). The present study was undertaken to characterize the signal transduction mechanisms underlying this serum deprivation-dependent activation of both the NHE1 and the concomitant invasive characteristics such as leading edge pseudopodia development and penetration of matrigel in breast cancer cell lines representing different stages of metastatic progression. Using pharmacological and genetic manipulation together with transport and kinase activity assays, we observe that the activation of the NHE1 and subsequent invasion by serum deprivation in metastatic human breast cells is coordinated by a sequential RhoA/p160ROCK/p38MAPK signaling pathway gated by direct protein kinase A phosphorylation and inhibition of RhoA. Fluorescence resonance energy transfer imaging of RhoA activity and immunofluorescence analysis of phospho-RhoA and NHE1 show that serum deprivation dynamically remodels the cell, forming long, leading edge pseudopodia and that this signal module is preferentially compartmentalized in these leading edge pseudopodia, suggesting a tight topographic relation of the signaling module to an invasion-specific cell structure.

INTRODUCTION

Tumor invasion and metastasis are the major causes of cancer deaths. The understanding of the mechanisms determining metastatic spread of malignant cells via invasion to distant tissues is a central question in oncology (Kurschat and Mauch, 2001). Metastatic progression is the result of a sequence of selective events that often involve interaction with elements of the tumor microenvironment (Rofstad, 2000; Bhujwalla *et al*., 2002), and it is important to identify the specific cellular and biochemical mechanisms that confer increased metastatic capacity under these conditions. There is increasing evidence that the acid component of the tumor microenvironment is an important determinant in controlling self-organized growth, invasive capacity, angiogenesis, and subsequent malignant progression (Martinez-Zaguilan *et al*., 1996; Gatenby and Gawlinski, 2001; Xu *et al*., 2002). The activity of the Na^+/H^+ exchanger isoform 1 (NHE1), the main regulator of intracellular and extracellular pH, has been shown to play a fundamental role in tumor cell motility (Reshkin *et al*., 2000; Lagana *et al*., 2000; Iwasaki *et al*., 2002) and invasion (Reshkin *et al*., 2000; Bourguignon *et al*., 2004; Paradiso *et al*., 2004).

The identification of the mostly still undefined tumor-specific signal transduction modules that are involved in driving or promoting metastatic progression is of particular importance (Cavallaro and Christofori, 2001; Ward *et al*., 2001). Recent advances in our understanding have highlighted the concept of intracellular organization of signaling complexes into cell/tissue- and/or stimulus-specific modules that give specificity to and increase efficiency of signal propagation (Hancock and Moon, 2000). Although much has been learned of the kinetics and action at all the levels of these cascades in normal cells, very little is known about their actual organization in neoplastic cells and their role in neoplastic processes and malignant progression. Our previous work highlighted a pivotal role for phosphoinositide 3-kinase (PI3K) and an antagonistic reciprocity between Rac1 and RhoA in the serum deprivation-dependent up-regulation of the Na^+/H^+ exchanger isoform NHE1 and consequent increased migratory and invasive capacities of human breast carcinoma cells (Reshkin *et al*., 2000; Paradiso *et al*., 2004). The full regulation of this event is probably orchestrated by a complex system or systems involving other signaling pathways of which the molecular components are unknown.

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04–10–0945) on April 20, 2005.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

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Abbreviations used: FRET, fluorescence resonance energy transfer; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; NHE1, Na^+/H^+ exchanger isoform 1.

The action of cAMP in the regulation of various cellular functions, including cell proliferation, differentiation, gene action, cytoskeleton, and movement, are well known (Skalhegg and Tasken, 2000). cAMP acts primarily through the activation of cAMP-dependent kinases (PKAs) that can transduce mitogenic signals from different growth factors and oncogenes, is overexpressed in the majority of human cancers, and has been implicated in positive, as well as negative, modulation of normal and malignant cell proliferation and apoptotic response (Lerner *et al*., 2000; Skalhegg and Tasken, 2000). Many studies have underlined the importance of PKA as a gating element in a number of different processes, including cancer motility and invasion (O'Connor *et al*., 1998; Korem *et al*., 1999; O'Connor *et al*., 2000; Dabizzi *et al*., 2003). There has recently been a paradigm change in the understanding of PKA-dependent regulation in which specificity is achieved in part by tight localization and temporal release/activation of signaling components at the functional subcellular compartment. There is also increasing evidence that PKA is a modulator of RhoA action in a large number of cellular regulatory contexts (Reshkin and Murer, 1991, 1992; Lang *et al*., 1996; Laudanna *et al*., 1997; Busca *et al*., 1998; Dong *et al*., 1998; Mukai *et al*., 2000; O'Connor *et al*., 2000; Forget *et al*., 2002; Murthy *et al*., 2003; Qiao *et al*., 2003). An important development in this field has been the recognition that the direct PKA-dependent phosphorylation of RhoA can alter its signal transduction function (Reshkin and Murer, 1991, 1992; Lang *et al*., 1996; Dong *et al*., 1998; Murthy *et al*., 2003). Currently, the significance of many of these interactions remains enigmatic, and it is a major challenge to establish their physiological and pathophysiological relevance and to dissect the diverse signaling pathways upon which and cellular compartment in which they act.

This study was undertaken to further investigate the signal transduction system(s) involved in the Na^+/H^+ exchanger-associated invasion in a well characterized human mammary epithelial cell line (MDA-MB-435) that represents the metastatic phase in progression (Cailleau *et al*., 1978; Price, 1996; Zhao *et al*., 2004) and has been shown to be highly metastatic in nude mice (Price *et al*., 1996). We observe that serum deprivation, a common tumor microenvironmental condition, dynamically remodels the cell, forming long, leading edge pseudopodia with a sorting of NHE1 and serine 188 phospho-RhoA to the distal tip together with a subsequent inhibition of RhoA activity and stimulation of NHE1 activity preferentially in these pseudopodia. This inhibits a downstream p160ROCK/p38 mitogen-activated protein (MAP) kinase signal transduction pathway, resulting in the stimulation of the NHE1 and subsequent invasion. These data were corroborated in other human breast cancer cell lines of different invasive potential and suggest that protein kinase A, RhoA, p160ROCK, p38 MAP kinase, and NHE1 are components of a novel signal transduction cassette activated in leading edge pseudopodia of breast cancer cells to overcome the normal physiological constraints arising from the common tumor microenvironmental condition of serum deprivation.

MATERIALS AND METHODS

Cells and Construction of Expression Vectors Containing RhoA Mutants

MDA-MB-435 cells, derived from a pleural effusion of a malignant human tumor; MCF-7 cells, derived from a primary, nonmalignant tumor; and MCF-10A cells, a spontaneously immortalized, untransformed human breast cell line derived from a patient with mild fibrocystosis, were cultured as described previously (Reshkin *et al*., 2000). MDA-MB-231 cells were cultured in the same way as the MDA-MB-435 cells. Relative cell length was analyzed by dividing, for 100 cells of each treatment, the cells greatest width by its greatest length (cell elongation index).

Site-directed mutagenesis on RhoA was performed by PCR overlap extension with the primers 5′-AAAAAGAATTCATGGCTGCCATCCGGAAGAAACTGGTG and 3′-AAAAAGTCGACTCACAAGACAAGGCAACCAGCTTTTTTCTTCCC to create $RhoA^{S188A}$ (Lang *et al*., 1996; Dong *et al*., 1998; Ellerbroek *et al*., 2003). The successful construction of the mutants was confirmed by DNA sequence analysis. The cDNA were cloned into the pBabe puro expression vector containing a hemagglutinin (HA) tag. Transient transfections were performed with the LipoTaxi reagent (Stratagene, La Jolla, CA).

Intracellular pH (pHi) and NHE1 Activity Determination

pHi was measured spectrofluorimetrically at 37°C with the fluorescent pH-sensitive probe 2′,7′-bis(carboxyethyl)-5(6′)-carboxyfluorescein, trapped intracellularly in cell monolayers grown on glass, and the activity of the Na^+/H^+ exchanger was measured by monitoring pHi recovery after an intracellular acid load produced with the NH_4Cl prepulse technique as described previously (Reshkin *et al*., 2000). After each experiment, trypan blue exclusion also was measured for each coverslip and when >5%, the experiment was not used.

In Vitro Assay for RhoA Activity

The vector pGEX4T-1 encoding the fusion protein for the Rho binding domain of the RhoA effector mDia, GST-mDia(RBD), which associates preferentially for GTP-bound RhoA, was obtained from Dr. Narumiya and analysis for active RhoA, i.e., that bound to GTP, was performed by a pull-down assay as described previously (Paradiso *et al*., 2004). The sample was extracted in 20 μl of 2× Laemmli buffer, run on 12% SDS-PAGE, and blotted onto Immobilon P (Millipore, Billerica, MA) for immunoanalysis with a RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

FRET Assays for In Vivo RhoA Activity

For these experiments, the Raichu 1297 probe (Yoshizaki *et al*., 2003; www.biken.osaka-u.ac.jp/biken/shuyouvirus/e-phogemon/) was used. In this sensor, the Rho binding domain (RBD) of the RhoA effector protein, Rhotekin, is sandwiched by VenusYFP and cyan fluorescent protein (CFP). The binding of endogenous GTP-RhoA to RBD generates a conformational change that displaces yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP), thereby decreasing fluorescence resonance energy transfer (FRET) between the two fluorophores. A reduction of intracellular active RhoA results in the opposite effect. In a parallel series of experiments, the activity balance between endogenous guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) was measured by imaging cells expressing the sensor Raichu-RhoA-1293 (Yoshizaki *et al*., 2003; www.biken.osaka-u.ac.jp/biken/shuyouvirus/e-phogemon/), which consists of a RBD (Rho binding domain of a RhoA effector protein)-truncated RhoA complex sandwiched between VenusYFP and CFP. With this probe, an increase in GEF activity increases the amount of GTP complexed to the RhoA moiety in the sensor, thereby increasing its intramolecular binding to RBD and bringing CFP in proximity to YFP, resulting in an increase in FRET. An increase in GAP activity induces the opposite effects. Twenty-four hours after transfection, monolayers of cells were cultured an additional 24 h in the presence or absence of serum and imaged on a Nikon ECLIPSE TE 2000-S equipped with a charge-coupled device camera, a controlled DeltaRAM monochromator on the excitation side and a beam splitter (Optical Insight, St. Cloud, MN) on the emission side fitted with a 505DCRX dichroic and two emission filters, D480/30 and D535/40. Excitation was at 430 nm and the dichroic mirror was a 455DRLP. Off-line image analysis was performed using the Metafluor 4.6 software (Meta Imaging 4.6; Universal Imaging, Downingtown, PA). Regions of interest (ROI) of 2 × 5 μm were fitted onto the cell in correspondence of either the cell main body or in the leading edge pseudopodia. All images were background subtracted. In measuring sensitized acceptor emission, that is, YFP emission at 545 nm upon excitation of CFP at 430 nm, spurious signal is collected due to direct excitation of YFP at 430 nm and by bleed-through of CFP emission in the yellow channel. To correct for such contaminating signals, we calculated net FRET (*nF*) as follows: $nF = I_{FRET} - (I_{YFP} \times a) - (I_{CFP} \times b)$, where I_{FRET} is sensitized YFP emission (excitation 430 nm, emission 545 nm) and I_{YFP} and I_{CFP} are YFP emission (545 nm) upon excitation at 480 nm and CFP emission (480 nm) upon excitation at 430 nm, respectively. *a* is a norm of the percentage of CFP bleed-through, and *b* is a norm of the percentage of direct excitation of YFP at 430 nm. *a* and *b* were determined by analyzing images of cells expressing only CFP or YFP as described previously (Xia and Liu, 2001), and for our system *a* and *b* values correspond to 64 and 8%, respectively. FRET ratio was calculated as I_{CFP}/nF.

p38 MAP Kinase Assay

After treatment, cells were washed with ice-cold phosphate-buffered saline (PBS), lysed 5 min at 4°C in ice-cold lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 20 mM Tris, pH 7.5)

plus 1 mM phenylmethylsulfonyl fluoride (PMSF), scraped into Eppendorf tubes, and triturated by sonification. The lysate was centrifuged at 4°C for 10 min at 14,000 rpm, and the supernatant was collected. Protein levels were normalizing to the protein levels measured before the assay. p38 MAP kinase activity was quantified using an immume complex kinase assay kit according to the manufacturer's protocol (New England Bioloabs, Beverly, MA). Total p38 expression measured by immunoblotting (Santa Cruz Biotechnology) was not found to vary under any experimental conditions (our unpublished data).

Cell Fractionation

After treatment monolayers were washed two times with ice-cold PBS and then lysed in ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM PMSF) and homogenated by five passes through a 20-gauge needle to obtain the total cell homogenate. An aliquot was removed for the determination of total cellular protein (Bradford method). The nuclear fraction was obtained by centrifuging the homogenate at 600 × *g* for 10 min. The resulting supernatant was centrifuged at 3500 × *g* for 10 min to obtain a pellet containing the endosomal fraction, and the supernatant was centrifuged again at 17,000 × *g* for 1 h to obtain a plasma membrane-rich pellet. All the separated cellular fractions were extracted with SDS sample buffer [6.25 mM Tris-HCl, pH 6.8, containing 10% (vol/vol) glycerol, 3 mM SDS, 1% (vol/vol) 2-mercaptoethanol, and 0.75 mM dibromophenol blue) and were run on 4–12% SDS-PAGE and analyzed by Western blotting.

Analysis of RhoA Serine Phosphorylation State

The amount of phosphorylated RhoA in the cell was measured by three independent methods. 1) After treatment, cell monolayers were washed twice with ice-cold PBS and lysed in ice-cold radioimmunoprecipitation assay (RIPA) plus 1 mM PMSF, 0.1% SDS, and 0.2% Na-deoxycholate. The cellular lysate was centrifuged at 14,000 rpm for 5 min at 4°C. Protein levels were normalized to the protein levels measured before the assay, and the supernatant was precleared with protein A-agarose for 2 h at 4°C. Cleared lysates were immunoprecipitated overnight at 4°C with phosphoserine antibodies conjugated to agarose (Sigma, Milan, Italy). The agarose beads were washed two times with RIPA plus 1% Triton X-100 and 0.2% Na-deoxycholate and three times with simple RIPA buffer. The pellet was resuspended in 50 μl of Laemmli buffer, run on 12% SDS-PAGE, and blotted onto Immobilon P (Millipore) for Western blotting analysis with a RhoA antibody (Santa Cruz Biotechnology). 2) In the cellular fractionation experiments, the phosphorlyation state of RhoA was analyzed by Western blotting by using first an anti-phosphoserine antibody (Sigma) followed by stripping of the blot and then Western blotting with the anti-total RhoA. Relative band density was quantified using the NIH ImageJ 1.29× software. 3) Phospho-RhoA location was analyzed by immunofluorescence with a polyclonal antibody against peptide of RhoA phosphorylated at serine 188 produced by PRIMM (Milan, Italy) and purified by immunoaffinity chromatography. Rabbits were immunized with a phosphoserine peptide containing the proximal 9 residues of RhoA, RRGKKK(pS)GC (Ellerbroek *et al*., 2003). Immunoprecipitation of aliquots of the same homogenates with each of the antibodies together with back-blotting with the other antibodies demonstrated that each of these techniques equally measure changes in in vivo RhoA phosphorylation state (Supplemental Figure 1).

Invasion

A quantitative measure of the degree of in vitro invasion of MDA-MB-435 cells was measured as the ability to traverse an 8-μm polycarbonate membrane coated with 5 μg of Matrigel (Chemicon International, Livermore, CA) as described previously (Reshkin *et al*., 2000). MDA-MB-435 cells were transfected with the indicated construct DNA or empty vector 48 h before the experiment and after 24 h were serum deprived or not for a further 24 h. The cells were then trypsinized, and 200,000 tumor cells were added in suspension to the upper chamber of the Boyden chamber in which the lower chamber contained 1% serum in culture medium. Culture dishes were returned to the incubator for 8 h, and then the cells that had traversed the filter were detached and measured according to the manufacturer's instructions. The fluorescent samples were read in a fluorescence plate reader at 480/520 nm (Cary Eclipse fluorescence spectrophotometer; Varian, Palo Alto, CA). Samples without cells but containing all other components were used as blanks. A standard curve of CyQuant dye binding to MDA-MB-435 cells was measured for each treatment. The number of MDA-MB-435 cells that had traversed the Matrigel layer was calculated using the standard curve of each treatment.

Protein Kinase A Assay

After treatment for the indicated times and concentrations, monolayers were washed twice with ice-cold PBS, scraped into ice-cold homogenization buffer (5 nM EDTA, 10 mM EGTA, 50 mM 2-mercaptoethanol, 1 mM PMSF, 10 mM benzamidine, and 50 mM Tris, pH 7.5), subjected to sonification, and the homogenate was centrifuged 30 min at 15,000 × *g* at 4°C. Protein content of the supernatant was measured by the Bradford method. PKA activity was evaluated in enzyme-linked immunosorbent assay by measuring the cAMP-dependent phosphorylation of an immobilized peptide substrate (RFARKGSLRQKNV) according to the manufacturer's (MBL, Nagoya, Japan) instructions.

Immunofluorescence

Cells plated on coverslips were fixed on ice in 3.7% paraformaldehyde/phosphate-buffered saline for 15 min and permeabilized for 10 min with 0.1% Triton X-100 in PBS and then blocked with 0.1% bovine serum albumin/0.2% gelatin in PBS for 1 h. Primary antibodies were diluted 1:50 for anti-RhoA (monoclonal; Santa Cruz Biotechnology), 1:100 for anti-phospho(serine188)RhoA (polyclonal; produced by PRIMM), and 1:100 for anti-NHE1 (Chemicon International) for 1 h. Secondary antibodies Alexa 488 goat anti-mouse IgG (for RhoA) and Alexa 568 goat anti-rabbit IgG [for phospho(serine188)RhoA and NHE1] conjugates (Molecular Probes, Eugene, OR) were diluted 1:1000 and incubated with the fixed cells for 1 h at room temperature. Coverslips were mounted with Vectashield medium (Vector Laboratories, Burlingame, CA) for anti-RhoA and anti-phospho(serine188)RhoA and with Vectashield plus 4,6-diamidino-2-phenylindole (DAPI) medium (Vector Laboratories) for anti-NHE1 and viewed on a Leica DMRXA microscope with a 40× objective. Images were obtained with the Nikon digital still camera DMX1200 by using ACT-1 software and processed identically in Adobe Photoshop.

RESULTS

PKA Positively and p38 Negatively Regulates Serum Deprivation-dependent Activation of NHE1 Activity

We had previously demonstrated that the serum deprivation-dependent stimulation of the NHE was specific to tumor cells and that 24 h of serum deprivation results in a potent but submaximal stimulation of the NHE1 without any decrease in cell viability (Reshkin *et al*., 2000). To determine which regulatory pathway(s) play a role in coordinating the stimulation of tumor cell exchanger activity by serum deprivation, we examined the effect of a panel of specific inhibitors/activators and/or dominant negative/active mutants of a large number of signaling kinases on the stimulation of NHE1 activity at 1 d of deprivation in MDA-MB-435 cells. This period of deprivation was chosen to optimize the stimulatory response and to minimize the time under pharmacological influence. After an initial screening of a number of signaling kinases (our unpublished data), we found that PKA and p38 MAP kinase were involved in regulating this process. Figure 1A illustrates a typical experiment measuring the rate of the NHE1-dependent pHi recovery (dpH_i/dt) after an acid load in serum-replete or -deprived MDA-MB-435 cells and in the presence or absence of 1 μM PKA inhibitor H89. Serum deprivation (trace 3) stimulated pHi recovery rate in these cells and H89, although having very little effect on nondeprived activity (trace 2), reversed this serum deprivation-dependent stimulation of basal NHE1 activity by >70% (compare trace 4 with trace 1). Figure 1B shows that stimulation of PKA with the adenylate cyclase activator, forskolin (solid bar), potentiated the stimulation of NHE1 activity in serum-deprived cells. Furthermore, Figure 1B shows that inhibition of p38 MAP kinase either by incubation with its specific inhibitor SB203580 (stippled bar) or by the transient expression of a dominant negative mutant (K>M) for p38α (striped bar) potentiated the stimulation of NHE1 activity. In line with these results, serum deprivation does stimulate the activity of PKA (Figure 1C) and inhibit the activity of p38 (Figure 1D). These data demonstrate that p38α MAP kinase negatively regulates serum deprivation-dependent activation of the tumor cell NHE1, whereas PKA positively regulates this activation.

Serum Deprivation Activates NHE1 via a Reduction in RhoA/p160ROCK Activity

Because PKA is a modulator of RhoA action in a large number of cellular regulatory contexts (see Introduction),

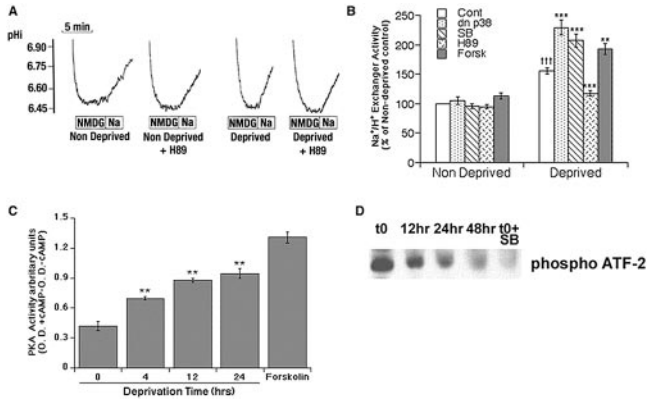

Figure 1. Inhibition of p38 MAP kinase and activation of PKA potentiate the serum deprivation-dependent stimulation of NHE1 activity. (A) Serum deprivation activates the NHE1 in MDA-MB-435 cells: typical experiment. After the dye loading procedure, cells were placed into the perfusion chamber and acidified by a 5-min pulse of 20 mM NH_4Cl. The trace begins at the start of perfusion of the monolayer with HEPES-NMEG solution, pH 7.4. There was no recovery of pHi under this condition. When the monolayer was perfused with 135 mM Na^+ nominally bicarbonate free-HEPES solution, pH 7.4, a rapid recovery of pHi commensed. The recovery in a control monolayer is compared with that in a series of monolayers incubated with 1 μM PKA inhibitor H89, for 24 h in the presence (NonDeprived) or absence (Deprived) of serum. (B) Summary of results: MDA-MB-435 monolayers were serum deprived or left in fresh complete growth medium for 24 h in the absence (empty bars) or presence of 1 μM H89 (cross-hatched bars), 1 μM PKA activator forskolin (stippled bars), 10 nM p38 inhibitor SB203580 (striped bars), or after the cells had been transfected with 10 μg of KRSPA-dnp38α (K>M) encoding a dominant negative mutant of the p38α isoform. NHE1 activity was measured, and data are the mean ± SE of between 15 and 20 observations for each condition. $^{+}p < 0.05$ and $^{+++}p < 0.001$ compared with nondeprived control, whereas $^{**}p < 0.01$ and $^{**}p < 0.001$ compared with Deprived control. PKA (C) or p38 (D) activity were measured at indicated times after serum deprivation as described in *Materials and Methods*. Data presented for the activity of p38 show one representative of four independently performed experiments. PKA data are the mean ± SE of between 6 and 10 observations for each condition. $^{**}p < 0.01$ serum-deprived cells compared with nondeprived (time 0) cells.

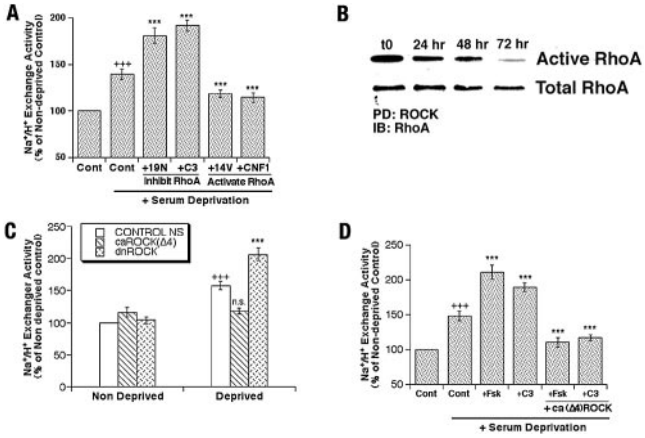

Figure 2. RhoA and p160ROCK positively regulate the serum deprivation-dependent stimulation of NHE1 activity. (A) MDA-MB-435 monolayers were exposed to serum deprivation after the cells had either been transfected with 10 μg of pBabe-dnRhoA (19N) vector encoding a dominant negative mutant of RhoA or pBabe-caRhoA (14V) encoding a constitutively active mutant of RhoA or treated with either the pharmacological RhoA inhibitor C3 exotoxin or the RhoA activator CNF1. The activity of the NHE1 was then measured as described in Figure 1. (B) Representative immunoblot of mDia(RBD) binding from four serum-deprivation time-course experiments. RhoA bound to the mDiaRBD (top) was normalized to the total RhoA content of cell extracts (bottom). (C) MDA-MB-435 monolayers were exposed to serum deprivation being transfected with 10 μg of construct. The activity of the NHE1 was then measured as in Figure 1. Western blot analysis with anti-FLAG confirmed the expression of the transfected genes. (D) Monolayers were transfected with constitutively active (Δ4)-p160ROCK or the empty pcDNA3(Flag) vector and 24 h later serum deprived in the absence or presence of Fsk or C3-exotoxin (C3) for 24 h. The activity of the NHE1 was measured as described in Figure 1. Data are the mean ± SE of between seven and 10 observations for each condition. $^{+++}p < 0.001$ compared with nondeprived control, whereas $^{***}p < 0.001$ compared with deprived control.

we next examined the role of RhoA in stimulated NHE1 activity in serum-deprived cells by using both pharmacological activators/inhibitors as well as dominant negative (dn) and constitutively active (ca) mutants. Inactivation of RhoA with either C3 exotoxin or the dn N19RhoA mutant potentiated the stimulation of NHE1 activity, whereas activating RhoA with either CNF-1 or the ca V14RhoA mutant reduced this stimulation by ~80% (Figure 2A). These data suggest that inhibition of RhoA activity is an integral part of the signal transduction module involved in the activation of the NHE1. To test this hypothesis, measurements of RhoA activity were made by analysis of its binding to a glutathione *S*-transferase (GST)-mDia(RBD) fusion protein that associates preferentially for GTP-bound RhoA (Kimura *et al*., 2000). These measurements verified that the activity of RhoA is reduced upon serum deprivation (Figure 2B) with a time course similar to that reported for the up-regulation of NHE1 activity by the same treatment (Reshkin *et al*., 2000).

RhoA-dependent regulation of NHE1 can be mediated by the serine/threonine kinase, p160ROCK, in a variety of cell types (Tominaga *et al*., 1998; Tominaga and Barber, 1998). To determine whether p160ROCK is the downstream RhoA effector in our experimental system, we measured the effect of transient transfection of dn (KDΔ 4) or ca (Δ 4) p160ROCK mutants (Itoh *et al*., 1999) in serum deprived cells on the activation of the NHE1. As observed for RhoA, the dn ROCK mutant (crossed hatched bar) potentiated the up-regulation of NHE1 whereas transfection with the ca ROCK mutant (striped bar) effectively blocked its up-regulation by >80% (Figure 2C). Furthermore, transient transfection with ca ROCK was able to completely block the potentiation of NHE1 activity obtained by either C3-exotoxin (or transient transfection with dn N19RhoA; our unpublished data) or by stimulation of PKA by forskolin (Fsk) (Figure 2D). These data demonstrate a positive regulatory role for RhoA through p160ROCK in the phenomenon of up-regulation of tumor cell NHE1 in serum-deprived cells and that the RhoA/ROCK complex is downstream of PKA.

Because MAP kinase action has been shown to be regulated by Rho family G proteins (Kyriakis and Avruch, 2001), we next examined whether PKA regulates stimulation of NHE1 activity through p38 MAPK and, if so, whether the RhoA-p160ROCK system is an intermediate between PKA and p38. The inhibitory effect of serum deprivation on the NHE1 by either H89 or transfection with ca 14VrhoA was reversed by concomitant inhibition of p38 with either SB203580 (Figure 3A) or by transfection with the dn (K>M) p38α mutant (our unpublished data), suggesting that p38 lies downstream of PKA, RhoA, and p160ROCK. In further

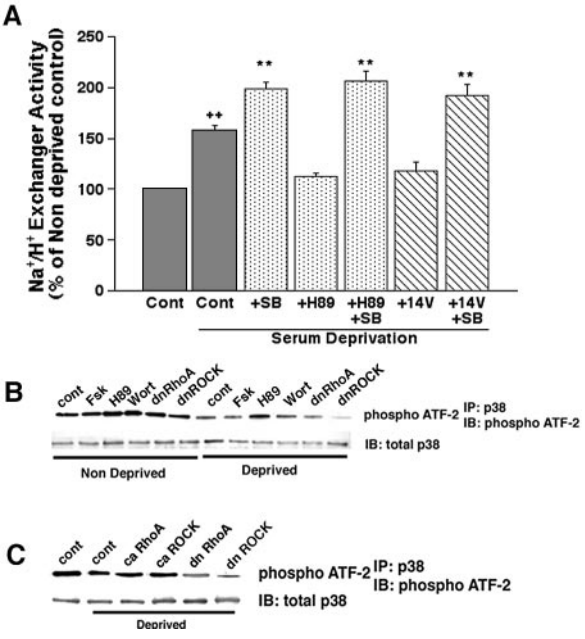

Figure 3. PKA and RhoA/ROCK are upstream of p38 in the regulation of serum deprivation-dependent stimulation of NHE1. (A) Both PKA-dependent and RhoA-dependent potentiation of serum deprivation-induced NHE1 activity are blocked by inhibiting p38. Monolayers were transfected with 10 μg of constitutively active RhoA (14V) or the empty pBabe vector and 24 h later serum deprived in the absence or presence of SB203580 (SB) and/or H89 for 24 h. The activity of the NHE1 was measured as described in Figure 1. Data are the mean ± SE of five to eight observations for each condition. $^{+++}p < 0.001$ compared with nondeprived control, whereas $^{**}p < 0.001$ compared with deprived control. (B) Serum deprivation-induced inactivation of p38 is dependent on PKA, RhoA, and p160ROCK. Monolayers were transfected with 10 μg of construct or their respective empty vector. After 24 h, cells were either serum deprived or left in the presence of serum in the absence or presence of Fsk, wortmannin (Wort), or H89 during the 24-h treatment. p38 MAP kinase activity was then measured as in Figure 1, and data presented are representative of four independently performed experiments. (C) Monolayers were transfected with 10 μg of construct or their respective empty vector. After 24 h, cells were either serum deprived or left in the presence of serum for an additional 24 h, and the activity of the p38 was measured as in Figure 1. Data presented for the p38 activity are representative of three independently performed experiments.

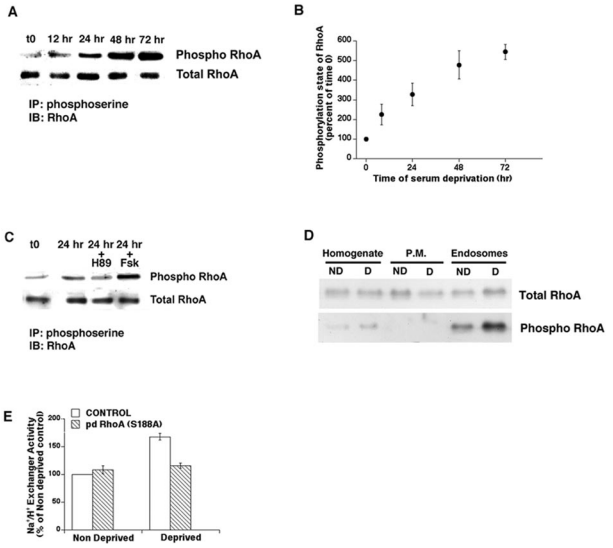

Figure 4. PKA-dependent phosphorylation of RhoA and role of phosphorylation at serine 188 in stimulation of NHE1 activity by serum deprivation. (A) Typical experiment in which lysates from cells deprived of serum for various times were subjected to immunoprecipitation with anti-phosphoserine antibodies, and the immunoprecipitates were probed with anti-RhoA (top) and normalized to total RhoA present in the lysate (bottom). (B) Densiometric analysis of RhoA phosphorylation relative to total RhoA expression. Mean of four independent experiments ± SE. (C) The effect of inhibition of PKA by incubation with H89 and activation of PKA by incubation with Fsk on RhoA phosphorylation state during the 24 h of serum deprivation. Representative blot of four independent experiments. (D) Typical experiment of the subcellular distribution of total RhoA and phospho-RhoA after 24-h serum deprivation (D) or 24-h serum-replete (ND) treatments. Here, serine phosphorylation of RhoA and total RhoA were measured directly on the blot using an anti-phosphoserine antibody followed by stripping of the blot and reprobing with the anti-RhoA antibody. Representative blot of three independent experiments. (E) Cell monolayers were transfected with phosphorylation dead S188A RhoA (striped bar), and after 24 h cells were either serum deprived or not for an additional 24 h at which time NHE1 activity was measured. Mean ± SE of four independent experiments.

support of these data, the serum deprivation-dependent decrease in p38 activity was reversed by H89, potentiated by forskolin, and dramatically potentiated by transfection of either dn N19RhoA or dn KDΔ 4ROCK (Figure 3B). In line with these data, both the ca Δ 4ROCK and ca V14RhoA blocked this serum deprivation-induced inhibition of p38 activity (Figure 3C). Interestingly, neither of the dominant negatives had any effect in the nonserum-deprived cells, demonstrating that this pathway functions specifically in the serum-deprived state.

PKA-dependent Phosphorylation of RhoA Is Increased upon Serum Deprivation and Is Necessary for the Up-Regulation of NHE1

As described above, serum deprivation-mediated activation of PKA and inactivation of a RhoA, ROCK, and p38 pathway lead to the subsequent activation of NHE1 in MDA-MB-435 cells. One mechanism by which PKA could gate this pathway is by down-regulating RhoA (Figure 2C) via the direct PKA-dependent phosphorylation of RhoA (Reshkin and Murer, 1991, 1992; Lang *et al*., 1996; Dong *et al*., 1998; Murthy *et al*., 2003). As seen in Figure 4, A and B, serum deprivation resulted in a dramatic increase in the phosphorylation of RhoA without changes in its expression level. The time course of this phosphorylation (Figure 4, A and B) was similar to that for both the inactivation of RhoA (Figure 2B) and the activation of the NHE1 (Reshkin *et al*., 2000). In serum-deprived cells, RhoA phosphorylation was potentiated by forskolin and inhibited by H89 treatment (Figure 4C), further supporting the suggestion that it is PKA-mediated. In concordance with other reports (Lang *et al*., 1996; Forget *et al*., 2002), cell fractionation experiments demonstrated that the phosphorylation of RhoA resulted in its removal from the plasma membrane and its translocation to the endosome fraction where the greatest increase in phosphorylation was observed (Figure 4D).

Whereas the preceding experiments indicate that RhoA is a substrate for PKA in these cells and that its phosphorylation is increased with serum deprivation, a critical question in the context of the current study is whether the observed PKA-dependent phosphorylation of RhoA is necessary for

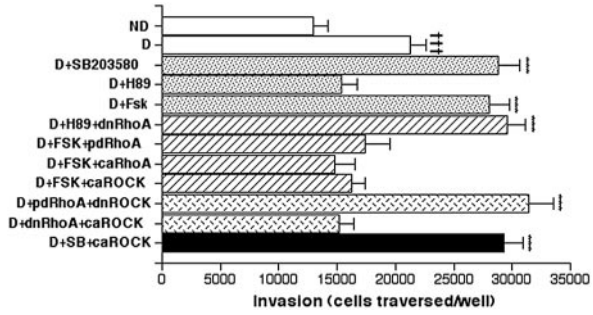

Figure 5. Invasion is regulated by serum deprivation with the same signal transduction module as NHE1. To examine the hierarchy of the above-described PKA-gated signal transduction module in regulating serum deprivation-induced invasive capacity, the ability of MDA-MB-435 cells to traverse a matrigel covered filter was measured as described in *Materials and Methods*. After having been transfected for 24 h with either the constructs or empty vector (empty bars) cells were deprived or not for the 24 h previous to the experiment. The number of invaded cells was determined as described in *Materials and Methods*. Mean ± SE, n = 16, $^{+++}p < 0.001$ compared with nondeprived control cells and $^{***}p < 0.001$ compared with deprived control cells.

the up-regulation of NHE1 activity. The phosphorylation of RhoA at serine 188 by PKA has been shown to block its action (Lang *et al*., 1996; Forget *et al*., 2002), suggesting that this serine could be the PKA target also in our case. Mutation of the PKA phosphorylation site serine188 to alanine to create a PKA phosphorylation dead (pd) RhoA mutant has been shown to abrogate this PKA-dependent regulation (Lang *et al*., 1996; Dong *et al*., 1998; Ellerbroek *et al*., 2003). Therefore, as an approach to assess the role of PKA-dependent phosphorylation of RhoA in the activation of NHE1, we mutated the PKA phosphorylation site serine188 to alanine to create a PKA pd RhoA mutant. Transfection of cells with this pd $RhoA^{S188A}$ mutant resulted in an abrogation of the up-regulation of the NHE1 (Figure 4E, striped bar), confirming the requirement for PKA-dependent phosphorylation of RhoA at serine 188 for regulation of NHE1 activity by serum deprivation.

Regulation of Invasive Capacity of MDA-MB-435 Cells Closely Follows That of Stimulation of the NHE1

We and others have observed that breast cancer cell-invasive capacity is tightly associated with the activity of the NHE1 and that specific inhibition of the NHE1 effectively blocks motility and invasion (Reshkin *et al*., 2000; Bourguignon *et al*., 2004; Paradiso *et al*., 2004). On the basis of these data, we analyzed invasive capacity after treatment with pharmacological agents and transient transfection with mutants to determine whether the above-described PKA-gated signal transduction module regulates invasive capacity with the same hierarchy as observed for its regulation of the NHE1. As seen in Figure 5, the ability of MDA-MB-435 cells to traverse a Matrigel-covered filter increased by ~60% after 24 h of serum deprivation. Inhibition of p38 with the specific inhibitor, SB203580 (first stippled bar), or activation of PKA with Fsk (third stippled bar) strongly potentiated this serum deprivation-induced increase in invasive potential. Incubation with the PKA inhibitor, H89 (second stippled bar), almost completely abrogated the serum deprivation-induced increase in invasive potential. Transfection with dn RhoA overcame the H89-dependent abrogation, whereas ca mutants of RhoA and p160ROCK and, importantly, the pd mutant of RhoA (serine 188 to alanine) blocked the ability of forskolin to stimulate invasion (stripped bars). Furthermore, neither pd RhoA nor dn RhoA was able to block the action of, respectively, either the dn or ca p160ROCK mutant (cross-hatched bars). Last, ca p160ROCK was not able to block the potentiation of serum deprivation-dependent invasive potential induced by incubation with SB203580 (solid bar). These data demonstrate that in the regulation of invasion the RhoA-ROCK complex is downstream of PKA and upstream of p38 just as was observed for the regulation of NHE1 activity. These data also support the hypothesis that the PKA-dependent phosphorylation of RhoA at serine 188 is a critical step in the stimulation of invasion by serum deprivation.

Serum Deprivation Leads to the Formation of Leading Edge Pseudopodia and the Localization of Phospho-RhoA and NHE1 and Inhibition of RhoA Preferentially in These Pseudopodia

Tumor cells have acquired altered morphological characteristics, leading edge pseudopodia, to facilitate their increased chemotaxis and invasive ability (Liotta and Clair, 2000; Taguchi *et al*., 2000). Our present results show that a signaling module involving PKA/RhoA/ROCK/p38/NHE1 is engaged in conditions of serum deprivation in metastatic cells that, as a consequence, show increased invasive behavior. Based on these observations, we hypothesize that serum deprivation may direct the selective activation of this signaling module at sites of pseudopodial extension where motility and invasion could be more efficiently controlled. To test this hypothesis, we first performed epifluorescence analysis by using specific antibodies against RhoA, phospho-(serine188)-RhoA and NHE1 before and after serum deprivation for 24 h (Figure 6A and Supplemental Figure 2). Serum deprivation resulted in the change from a generally fusiform (mesenchymal) shape to the formation of long, leading edge pseudopodia (cell elongation index of 4.86 ± 0.19 vs. 10.35 ± 0.93 in nonserum-deprived and -deprived cells, respectively, n = 100, $p < 0.001$). This change in shape was accompanied by a shift from a generally localized distribution of all three proteins in the serum-replete condition (NonDeprived) to the preferential localization to the leading edge pseudopodia (arrowheads) during serum deprivation.

To further explore the possible redistribution and localization of this signaling module to the leading edge pseudopodia of MDA-MB-435 cells, we compared the location, activity, and regulation of RhoA in MDA-MB-435 cells when subjected to serum deprivation. RhoA activity was measured in whole cells by using a FRET-based sensor, Raichu 1297 in which the RBD RhoA effector protein, Rhotekin, is sandwiched by VenusYFP and CFP. With this sensor, a reduction in the concentration of active RhoA results in a higher fraction of the probe where CFP and YFP are far apart, resulting in a lower value of FRET ratio (see *Materials and Methods*). In basal conditions, MDA-MB-435 cells were characterized by a 18.5 ± 1.3% higher RhoA activity in pseudopodia (p) than in the main body (mb) (n = 41, $p <$ 0.001 for paired data). Spatial analysis of the decrease of RhoA activity (Figure 6B), revealed a stronger inhibition of RhoA due to serum deprivation in the pseudopodia (–12.4 ± 4.1 vs. −36.5 ± 5.7%, $p < 0.001$, in the main cell body and in the pseudopodia, respectively). Indeed, their relative activity was reversed such that after serum deprivation the main body had a 11.5 ± 2.2% higher RhoA activity than the pseudopodia. Importantly, inhibition of PKA with the specific inhibitor H89 (1 μM), during the 24 h of serum deprivation (Figure 6B) did not significantly change RhoA activity

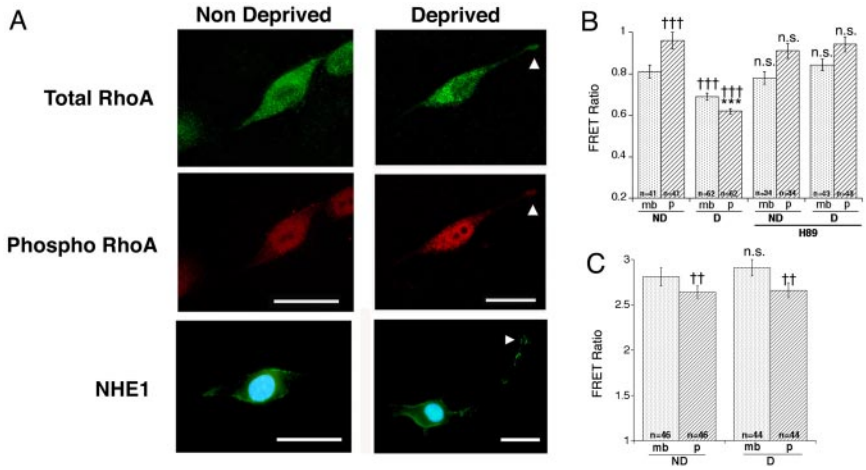

Figure 6. Effect of serum deprivation on cellular compartmentalization of total-RhoA, phospho-RhoA, and NHE1 expression and RhoA activity in MDA-MB-435 cells. (A) Epifluorescence images of colabeled total RhoA and phospho-RhoA and of NHE1 in MDA-MB-435 cells treated for 24 h in serum replete (NonDeprived) or deprived medium. Note the change in cell shape with the formation of leading edge pseudopodia and redistribution of the total and phospho-forms of RhoA and of NHE1 to these pseudopodia (arrowheads) after serum deprivation. In images of NHE1, the nucleus has been colored with DAPI. Bar, 10 μm. (B) Cells were transfected with a plasmid containing the RBD of the RhoA effector protein Rhotekin, sandwiched by VenusYFP and CFP (Raichu 1297x) as described in *Materials and Methods*. RhoA activity: comparison between the FRET ratio change in response to 24-h serum deprivation (−serum) ± the presence of the PKA inhibitor H89 in the main body (mb) and the pseudopodial protrusions (p) in MDA-MB-435 cells. A decrease in FRET ratio signifies a decrease in RhoA activity. n varied from 34 to 62, $^{+++}p < 0.001$ compared with nondeprived control cells and $^{***}p < 0.001$ compared with main body of deprived cells. n.s., not significant. (C) RhoA regulation by GEF and GAP proteins. Cells were transfected with a plasmid containing RhoA sandwiched by VenusYFP and CFP (Raichu-RhoA-1293 ×) as described in *Materials and Methods*. Comparison between the FRET ratio change in response to 24-h serum deprivation (−serum) in the main body (mb) and the pseudopodial protrusions (p) in MDA-MB-435 cells. n varied from 34 to 62, $^{++}p < 0.01$ compared with nondeprived control cells. n.s., not significant.

in nondeprived conditions, whereas it provoked a total reversal of the RhoA inhibition that had been promoted by serum starvation. Moreover, to exclude a role of a change in equilibrium between GEF and GAP regulatory activities in regulating RhoA in serum-deprived conditions, we transfected cells with the Raichu-RhoA-1293 × probe that monitors the activity balance between GEFs and GAPs. As shown in Figure 6C, there was no significant difference in the FRET ratio of this probe between serum-starved and normal conditions supporting the suggestion that the observed reduction in RhoA activity with serum deprivation is due only to its phosphorylation by PKA. In normal, nontumor cells (MCF-10A), the RhoA response to serum deprivation occurred evenly throughout the cell and was independent of PKA activity (Supplemental Figure 3). Together, these data further support the hypothesis that in cancer cells serum deprivation locally activates a PKA/RhoA-controlled signaling module organized within an invasion-specific cell structure that leads to a more efficient invasive behavior/phenotype.

This Signal Module in Other Human Breast Cancer Cells

We finally examined whether our findings apply to other human breast cancer cell lines with different invasive capacities. Analysis of the phosphorylation state of RhoA in cellular homogenates by using the phospho(serine188)-RhoA antibody and a general anti-phosphoserine antibody demonstrated that serum deprivation reduced RhoA phosphorylation in the normal, MCF-10A cells and increased RhoA phosphorylation in both the primary carcinoma, low invasive cell line MCF-7 and the highly invasive, metastatic MDA-MB-231 cells (Supplemental Figure 4). We then analyzed the role of PKA, RhoA, and p38 in regulating NHE1 activity in the MCF-7 and MDA-MB-231 cell lines (Figure 7A). In both cell lines, H89 inhibition of PKA blocked the serum deprivation-dependent stimulation of NHE1, whereas the SB203580 inhibition of p38 potentiated this stimulation, and simultaneous inhibition of both kinases displayed the same pattern as the inhibition of p38 by SB203580 alone (Figure 7A, stippled bars). These data demonstrate that PKA-dependent regulation of NHE1 activity is upstream of p38 also in these cell lines. Importantly, transfection of the phospho-dead RhoA mutant completely blocked the stimulation of NHE1 by deprivation in both cell lines, verifying the fundamental role of RhoA phosphorylation in that process (Figure 7A, stripped bars). In line with these results, serum deprivation produced a remodeling of both cell lines with both RhoA and phospho-RhoA being strongly expressed in the tip of the pseudopodia (Figure 7B) and preferentially induced a PKA-dependent inhibition of RhoA in the pseudopodia of both cell lines (Figure 7C), as was observed for MDA-MB-435 (Figure 6).

DISCUSSION

In the present study, we have characterized the signal transduction system regulating the serum deprivation-dependent stimulation of the NHE1 in the advanced breast carcinoma cell lines MDA-MB-435 and MDA-MB-231 and in the less invasive MCF-7 cell line derived from a primary tumor. We describe a novel signal transduction module localized in dominant leading edge pseudopodia of breast cancer cells that, during serum deprivation, integrates PKA, RhoA, p160ROCK, and p38α into a phosphorylation-triggered signal cascade hierarchy that controls tumor cell NHE1 activity and invasive capacity. PKA acts through phosphorylation of RhoA on serine 188 to release the suppression of NHE1 activity by the RhoA/p160ROCK/p38α pathway (Figure 8).

Many studies have underlined the importance of PKA as a gating element in a number of different signaling systems and an important example of this is the gating of integrin-stimulated invasion in human breast (O'Connor *et al*., 1998), colon (O'Connor *et al*., 2000) cancer cells and in luteinizing hormone regulation of human endometrial cancer cells (Dabizzi *et al*., 2003), demonstrating a key regulatory role for PKA in the cancer invasive process. RhoA is known to be overexpressed in breast cancer cells and to play a role in increased invasion (Lin and van Golen, 2004), and one PKA gating mechanism in normal cells is the PKA phosphorylation of RhoA on Ser188, which leads to its increased removal

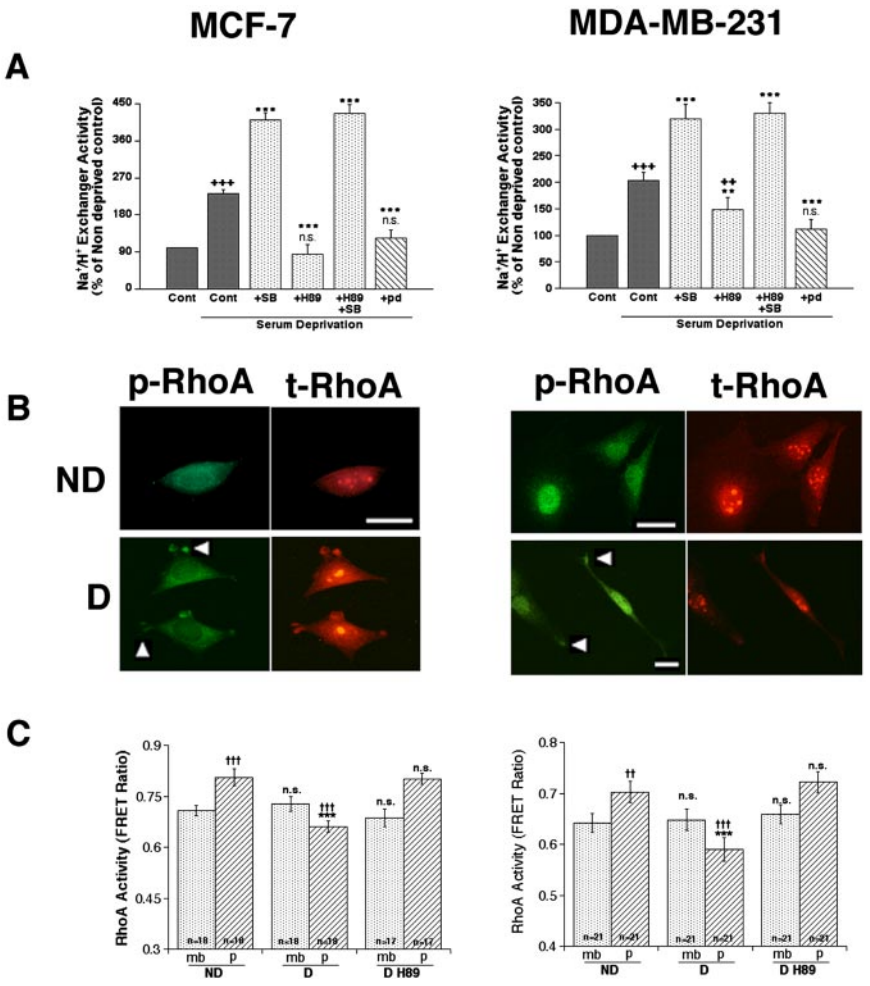

Figure 7. Role of PKA/RhoA/ROCK/p38 signal module in serum deprivation-dependent regulation of NHE1 and cell shape in MCF-7 (left) and MDA-MB-231 (right) breast cancer cell lines. (A) PKA-dependent potentiation of serum deprivation-induced NHE1 activity is blocked by inhibiting p38 and role of phosphorylation at serine 188 in stimulation of NHE1 activity by serum deprivation in both MCF-7 and MDA-MB-231 cell lines. Experiments were conducted as described in Figure 3 and 4 legends. Data are the mean ± SE of between nine and 12 observations for each condition. $^{+++}p < 0.001$ and $^{++}p < 0.01$ compared with nondeprived control, whereas $^{**}p < 0.001$ and $^{**}p < 0.01$ compared with deprived control. (B) Epifluorescence images of colabeled total RhoA and phospho-RhoA cells in MCF-7 and MDA-MB-231 cells treated for 24 h in serum replete (NonDeprived) or deprived medium. Arrowheads indicate pseudopodial-located phospho RhoA and RhoA. (C) Serum deprivation induces PKA-dependent inhibition of RhoA activity in the pseudopodia of both MCF-7 and MDA-MB-231 cells. Experiments were conducted as described in Figure 6B legend, and the number of experiments is indicated in their respective bars. $^{+++}p < 0.001$ and $^{++}p < 0.01$ compared with nondeprived control cells and $^{***}p < 0.001$ compared with main body of deprived cells. n.s., not significant.

from the plasma membrane (Lang *et al.*, 1996; Forget *et al.*, 2002) and to a decreased association with its downstream effector p160ROCK (Laudanna *et al.*, 1997; Busca *et al.*, 1998). Whereas PKA and RhoA have been shown to have opposite effects on cancer cell migration (O'Connor *et al.*, 2000), our results demonstrate for the first time in cancer cells the importance of direct PKA-dependent phosphorylation and inhibition of RhoA in the invasive process.

The orchestration of complex cellular events, such as motility or invasion, involves the assembly of multimolecular complexes or modules at the cellular site of action (Hancock and Moon, 2000). It is now well established that tumor cells have acquired altered morphological characteristics to facilitate their increased invasive ability (Lagana *et al.*, 2000) and establishment of the directed cell polarity involved in invasion requires dynamic remodeling of the cytoskeleton and sorting of proteins to the leading-edge pseudopodial compartment. Serum deprivation provoked an extension of the leading edge pseudopodia, a compartment that has been previously identified to be involved in cancer cell invasion (Liotta and Clair, 2000; Taguchi *et al.*, 2000). This serum deprivation-dependent extension was accompanied by a redistribution of NHE1, RhoA and an increase in phospho-RhoA along the trunk and, especially strong, at the distal tip (Figures 6A and 7C). Furthermore, we observe here that in the cancer cell serum deprivation provokes an inhibition of RhoA activity preferentially in the leading edge pseudopodia that is totally dependent on its phosphorylation by PKA

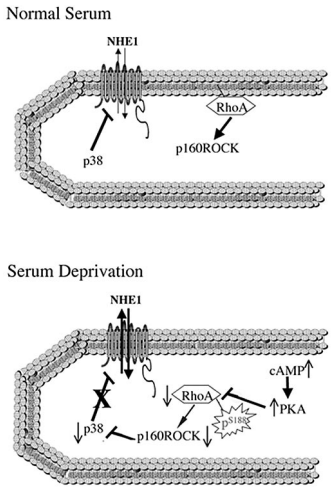

Figure 8. Model of serum deprivation-induced signal transduction module that activates the NHE1 and promotes invasion in breast cancer cells. A reduced level of serum in the tumor metabolic microenvironment increases the activity of PKA and induces a shift from a signal transduction module that excludes PKA phosphorylation of RhoA to another in which this phosphorylation is possible. This phosphorylation inhibits the activity of RhoA, resulting in the subsequent inhibition of p160ROCK and p38 MAP Kinase. The down-regulation of this NHE1 repressor signaling module results in the stimulation of NHE1 activity, the formation of a leading edge pseudopodia and subsequent invasive capacity.

(Figures 6, B and C, and 7D), whereas in normal, nontumor cells (MCF-10A) the RhoA response to serum deprivation occurs evenly throughout the cell and was independent of PKA activity (Supplemental Figure 3). Together, the data presented here support the idea that in breast cancer cells serum deprivation locally activates a PKA controlled signaling module organized within an invasion-specific cell structure that leads to a more efficient invasive behavior. We hypothesize that the protein clustering and inhibition of RhoA in the leading edge pseudopodia is necessary for its formation and for invasion (Figure 8). This association of increased motility with an inhibition of RhoA activity is similar to that recently reported for the tight junction protein NZO-3 in controlling renal cell motility (Wittchen *et al*., 2003), inhibition of motility, and invasion by dihydromotuporamine C (McHardy *et al*., 2004), tenascin-induced colon cancer cell invasion (De Wever *et al*., 2004) or by the degradation of RhoA by Smurf1 in controlling transformed Mv1Lu and HEK293T cell motility (Wang *et al*., 2003), suggesting a common mechanism. This last work observed RhoA degradation primarily in the cell protrusions supporting our present results concerning the loss of RhoA activity, although the underlying mechanism is different. Interestingly, localized activation of a RhoA/ROCK/p38MAPK signaling module to the pseudopodial domain of invasive Moloney sarcoma virus-transformed epithelial cells (MSV-MDCK-INV) regulates NHE1-dependent pseudopodial extension and motility (Jia, Noël, and Nabi, personal communication). Together, these data suggest that the RhoA/ROCK/p38MAPK signal module may be common to transformed/cancer cell regulation of invasive potential by various extracellular signals. Intriguingly, a similar inhibition of the RhoA/ROCK pathway has been reported in controlling the process of neurite outgrowth (Busca *et al*., 1998; Luo, 2000; Scaife *et al*., 2003; Yuan *et al*., 2003; Kishida *et al*., 2004), and in the latter article, it was serum deprivation that induced neuroblastoma neurite outgrowth dependent on the inhibition of RhoA. These data suggest that neurite outgrowth has profound similarities to the tumor-invasive process.

The data presented in this article are important in the context of the critical role of subcellular localization and timing of PKA signaling in determining specificity of downstream regulatory processes (Zaccolo *et al*., 2002; Tasken and Aandahl, 2004). This difference in PKA-dependent signaling is of extreme interest clinically because studies have demonstrated both positive as well as negative cAMP/PKA modulation of tumor cell proliferation and apoptotic response, thereby rendering a unified therapeutic approach difficult (Lerner *et al*., 2000; Skalhegg and Tasken, 2000). A possible explanation for these seemingly contradictory reports concerning PKA regulation may be that the pleiotrophic action of PKA in cell function is based on the fine control of its spatiotemporal regulation. In this context, we recently demonstrated that treatment of these same breast cancer cells with the antineoplastic agent paclitaxel induces apoptosis via a direct PKA-induced stimulation of p38 MAP kinase, resulting in inhibition of NHE1 and subsequent induction of apoptosis (Reshkin *et al*., 2003). This could activate an alternative PKA-dependent signal module that differently regulates p38. The relative relationship between the last two downstream components of each signaling module remained the same: an increase in p38 activity inhibited the NHE1, whereas a decrease in p38 activity stimulated the NHE1.

Our results are consistent with a model in which the local cAMP pulse initiates a shift from a signal transduction module excluding PKA phosphorylation of RhoA to another in which this phosphorylation is possible. We suggest that this could be due to an altered expression/distribution of a PKA anchoring and/or a scaffolding protein. These protein modules are organized into physically and functionally distinct units that can reorganize when cell conditions change (Yaffe and Cantley, 1999). Ezrin is an actin-binding and PKA-anchoring protein that has been shown to also bind to NHE1 as a part of NHE1-dependent regulation of actin cytoskeletal organization and motility (Denker *et al*., 2000). RhoA through p160ROCK has been demonstrated to regulate both NHE1 activity (Tominaga *et al*., 1998) and ezrin phosphorylation (Shaw *et al*., 1998) and location (Kotani *et al*., 1997). The ezrin binding protein 50 (or NHERF1) is a scaffolding protein known to link ezrin to various proteins and thereby regulate their PKA phosphorylation state (Voltz *et al*., 2001). Together, these studies suggest that ezrin and NHERF could play important roles in the assembly and regulation of the serum deprivation-dependent signaling module described in the present study.

In conclusion, our results demonstrate that serum deprivation up-regulates the activity of tumor cell NHE1 via a novel PKA-gated signal transduction module that is locally activated in leading edge pseudopodia. These data demonstrate that tumor cells have developed different regulatory patterns that allow them to adapt to and take advantage of the specialized conditions of their microenvironment to maximize their neoplastic potential. The preferential activation of this invasion-specific signal module in the cellular structure specific for invasion, the pseudopodia, contributes further to the identification of the biochemical/structure-determined complexes fundamental for the digestion and infiltration of surrounding tissues by the cancer cell.

ACKNOWLEDGMENTS

We thank Dr. P. P. Di Fiore (European Institute of Oncology, Milan, Italy) for the dn RhoA plasmid; Dr. Itoh for the dn and ca p160ROCK plasmids; Dr. M. H. Symons (Picower Institute for Molecular Research, Manhasset, NY) for the ca RhoA plasmid; Prof. S. Narumiya (Kyoto University, Kyoto, Japan) for the GST-mDia(RBD) plasmid; Prof. M. Matsuda (Osaka University, Osaka, Japan) for the Raichu 1297x and 1293x plasmids; and Dr. C. Fiorentini (Istituto Superiore di Salute, Rome, Italy) for the CNF1 toxin. This work was supported by PSO Consiglio Nazionale delle Ricerche-Ministero dell'Istruzione, dell'Università e della Ricerca Grant CU03.00304 and Fondo per gli Investimenti della Ricerca di Base Grant RBAU01B3A3-001. We thank Centro di Eccellenza di Genomica in Campo Biomedico ed Agrario for providing the FRET microscope. A. B. is the recipient of a fellowship from Italian Federation for Cancer Research. M. Z. is supported by Telethon Italy (TCP00089), the European Commission (QLK3-CT-2002-02149), the Fondazione Compagnia di S. Paolo, and the Italian Cystic Fibrosis Research Foundation.

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